

Duplicate chlorophyll-deficient loci in soybean

K.K. Kato and R.G. Palmer

Abstract: Three lethal-yellow mutants have been identified in soybean (*Glycine max* (L.) Merr.), and assigned genetic type collection numbers T218H, T225H, and T362H. Previous genetic evaluation of T362H indicated allelism with T218H and T225H and duplicate-factor inheritance. Our objectives were to confirm the inheritance and allelism of T218H and T225H and to molecularly map the locus and (or) loci conditioning the lethal-yellow phenotype. The inheritance of T218H and T225H was 3 green : 1 lethal yellow in their original parental source germplasm of *Glycine max* 'Illini' and *Glycine max* 'Lincoln', respectively. In crosses to unrelated germplasm, a 15 green : 1 lethal yellow was observed. Allelism tests indicated that T218H and T225H were allelic. The molecular mapping population was *Glycine max* 'Minsoy' × T225H and simple sequence repeat (SSR) markers were used. The first locus, designated *y18_1*, was located on soybean molecular linkage group B2, between SSR markers Satt474 and Satt534, and linked to each by 4.4 and 13.4 cM, respectively. The second locus, designated *y18_2*, was located on soybean molecular linkage group D2, between SSR markers Satt543 and Sat_001, and linked to each by 2.2 and 4.4 cM, respectively.

Key words: duplicate gene, *Glycine max*, homoeologous genomic segment, genome evolution, lethal-yellow mutant.

Résumé : Trois mutants jaunes létaux ont été identifiés chez le soja (*Glycine max* (L.) Merr.) et désignés T218H, T225H et T362H. Une caractérisation génétique antérieure avait indiqué que T362H était allélique à T218H et T225H, tout en produisant une hérédité typique de gène dupliqués. Les objectifs des auteurs étaient de confirmer l'hérédité et l'allélisme de T218H et de T225H ainsi que de réaliser la cartographie génétique des locus déterminant ce phénotype à l'aide de marqueurs moléculaires. La progéniture des sources parentales de T218H et de T225H, les cultivars *Glycine max* 'Illini' et *Glycine max* 'Lincoln,' respectivement, montrait une ségrégation 3 verts : 1 jaune létaux. Suite à des croisements avec des lignées non-apparentées, une ségrégation 15 verts : 1 jaune létaux a été observée. Des tests d'allélisme ont montré que T218H et T225H étaient alléliques. Une population de cartographie génétique a été produite à partir du croisement *Glycine max* 'Minsoy' × T225H et des marqueurs microsatellites (SSR) ont été employés. Le premier locus, appelé *y18_1* a été localisé sur le groupe de liaison B2 entre les marqueurs Satt474 et Satt534, à une distance de 4,4 et de 13,4 cM, respectivement. Le second locus, *y18_2*, a été situé sur le groupe de liaison D2 à 2,2 cM de Satt543 et à 4,4 cM de Sat_001.

Mots clés : gène dupliqué, *Glycine max*, segment génomique homéologue, évolution génomique, mutant jaune létaux.

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Introduction

A balance between gene duplication and gene loss has fundamentally shaped plant genomes. Gene duplication occurs at three levels, whole genome polyploidization, segmental duplications, and single-gene duplication. Polyploidization is widespread in plants, with an estimated frequency of 30% to 80% (Masterson et al. 1994). Recent genomic analyses indicate that 50% to 70% of angiosperms have experienced one or more episodes of chromosome doubling in their evolutionary history (Ku et al. 2000; Mayer et

al. 2001; *Arabidopsis* Genome Initiative 2000; Wendel 2000). Based on genomic and single-gene approaches, the modern angiosperm genome is characterized by a series of nested duplications of varying age. In *Arabidopsis*, most of the 25 500 genes are found in 103 duplicated blocks, and about 17% of these genes are arranged in tandemly repeated segments (*Arabidopsis* Genome Initiative 2000; Vision et al. 2000). Duplicate genes and (or) genomes have been reorganized through processes such as loss of duplicated segments, single-gene duplication via unequal crossing over, chromosome repatterning, transposition, gene conversion, recombination, and divergent evolution (Matzke and Matzke 1998; Durbin et al. 2000; Ohta 2000; Zhang et al. 2001). In addition to genome restructuring, gene-level changes like gene silencing also occur after gene duplication. In some polyploids, chromosomal reorganization and gene silencing ("diploidization") are so extensive that the genome is no longer structured as an allopolyploid (Soltis and Soltis 1999).

Soybean has been suggested to be an ancient polyploid based on chromosome numbers (Lackey 1980; Bruneau et al. 1994), genome size (Arumuganathan and Earle 1991), and the identification of duplicate loci, homoeologous regions, in which several pairs of genes with similar or identi-

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cal function are located (Shoemaker et al. 1996). In addition, presumably earlier gene duplication in the soybean genome was suggested by the genomic analysis of duplicate functional genes, *Pal* and *Pa2*, via *Arabidopsis* genome sequences (Lee et al. 1999, 2001).

Three lethal-yellow mutants, genetic type collection numbers T218H, T225H, and T362H, have been identified in soybean (Palmer et al. 2004). T218H was inherited as a single-gene recessive trait in 'Illini' (Palmer 1987) and originally was from the mutable form T218M.

The soybean mutable chlorophyll allele *Y18-m* can mutate either to stable conditions, *Y18* or *y18*, or to different states of instability (Peterson and Weber 1969). When *Y18-m Y18-m* plants were grown at 19 or 29 °C, more total mutant sectors and greater total mutant area were found at 19 °C than at 29 °C (Sheridan and Palmer 1977). However, more total yellow tissue and more yellow tissue per sector were found at 29 °C than at 19 °C, indicating that the timing of mutation from *Y18-m* to *y18* was affected by temperature. Earlier mutation of *y18* at 29 °C resulted in fewer, larger sectors of yellow tissue. The lethal-yellow mutant was inherited as a single-gene recessive trait and was assigned genetic type collection number T225H (Sheridan and Palmer 1975).

Somaclonal variation has been reported in many plant species, including soybean. Organogenic and embryogenic callus cultures (Amberger et al. 1992a; Barwale and Wildholm 1987; Hawbaker et al. 1993) and cotyledonary node cultures (Freitag et al. 1989; Graybosch et al. 1987) have been used in soybean in an attempt to produce heritable genetic variation. Amberger et al. (1992b) reported that the Chinese cultivar *Glycine max* 'Jilin 3' (PI 427099) was very conducive to the generation of somaclonal variation. A single chimeric (variegated trifoliolate) plant was observed in the R₃ generation in a progeny row of tissue culture derived 'Jilin 3'. Upon self-pollination of this chimera, the progeny were classified as green foliage, viable yellow, lethal yellow, and chimeric foliage. The lethal-yellow mutant was inherited as a single-gene recessive trait in 'Jilin 3' and was allelic to *y18* (Palmer et al. 2000). This lethal-yellow mutant was assigned genetic type collection number T362H and gene symbol *Y18y18* (Ames 2) (Palmer et al. 2000).

The objectives of this research were to determine the inheritance and allelism of the three lethal-yellow mutants and to clarify the genomic regions containing the chlorophyll-deficient mutant loci for the study of soybean genome evolution.

Materials and methods

The soybean genetic type collection comprises strains with qualitative genetic traits. These strains are assigned T numbers. For T strains with an H suffix (e.g. T218H), the allele is carried as the heterozygote, because the homozygote is lethal or very weak. For T strains with an M suffix (e.g. T218M), the trait is maintained by selecting the mutable genotype.

Genetic analysis

Genetic study

Three *y18* lethal-yellow mutants are maintained in the United States Department of Agriculture (USDA) Genetic

Type Collection: T218H, T225H, and T362H (Palmer et al. 2003). Inheritance tests with these mutants were done by crossing chimeric plants (T218M; *Y18-m*) as male parent to *Glycine max* 'Illini' (*Y18 Y18*) to derive T218H, (*Y18 y18*) (Palmer 1987), and by crossing growth chamber grown lethal-yellow plants of T225 (*y18 y18*) as male parent with *Glycine max* 'Lincoln' (*Y18 Y18*) to derive T225H (Sheridan and Palmer 1975). The lethal-yellow plants (*y18 y18*) can be grown in a growth chamber under reduced light to flowering and sometimes to maturity. The inheritance tests would be for segregation of green plants and lethal-yellow plants, T218 and T225, in their original source genetic backgrounds of 'Illini' and 'Lincoln', respectively. There would be no chimeric or mutable plants.

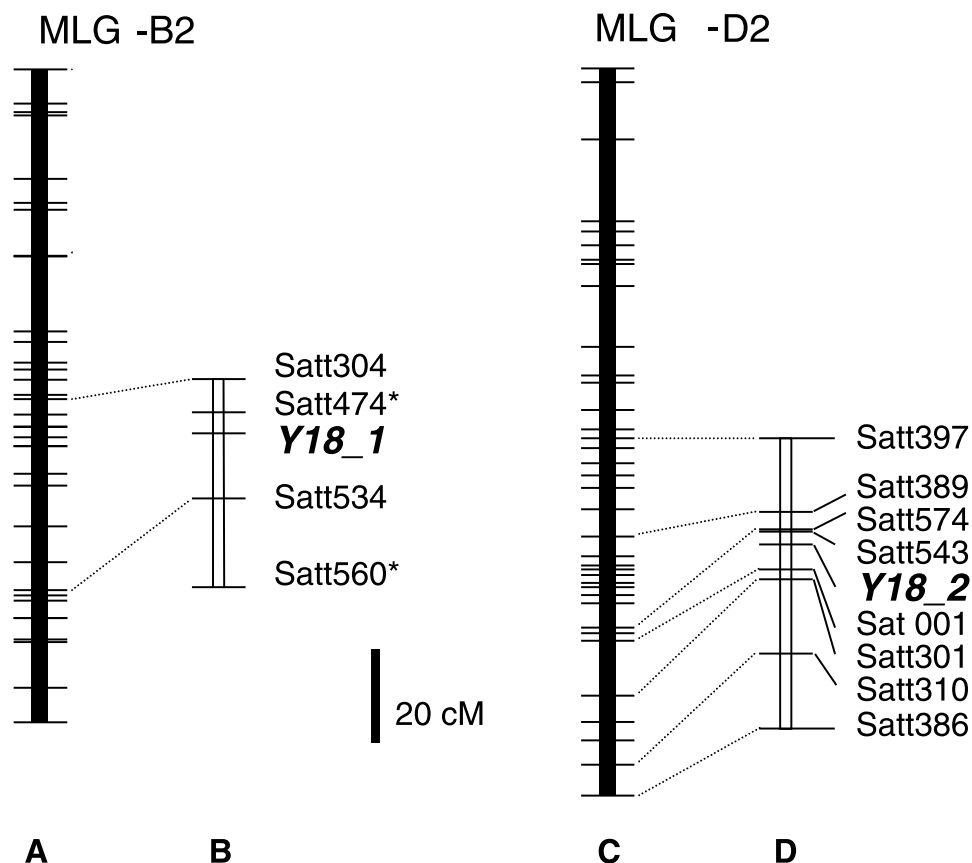
To determine the inheritance of T218 (*y18 y18*) in a different genetic background than the original source, 'Illini', green plants in segregating T218H families were crossed reciprocally to PI 437477B (*Y18 Y18*). The F₂ data from different F₁ plants and from the reciprocal crosses were tested for homogeneity. The homogeneity χ^2 was non-significant and the data were pooled. To determine the inheritance of T225 in a different genetic background than the original source, 'Lincoln', green plants in segregating T225H families were crossed as female parent to *Glycine max* 'Minsoy' (PI 27890) and to PI 437477B. Progeny testing of green plants in parental T218H and T225H families identified the heterozygotes (*Y18 y18*). Data were collected from F₂ plants and from F₂-plant progeny rows. 'Minsoy' and PI 437477B have the dominant trait, sharp pubescence tip, which can be classified on seedlings (leaves) or mature plants (stems and pods). Hybrid plants can be recognized as seedlings. These two parents were used because soybean is considered a paleo-hexaploid (Shoemaker et al. 1996). In soybean, single-gene recessive traits in the original source background have segregated as duplicate-factor traits in unrelated backgrounds, e.g. brachytic stem (Kilen 1977; Boerma and Jones 1978), and a male-sterile, female-sterile mutation (Ilarslan et al. 1997).

Allelism tests with T218H and T225H were done by making reciprocal cross-pollinations of green plants in entries segregating for green (viable) and lethal-yellow plants. Genotype of the green parent plants was determined by progeny testing. Data were collected from F₁ and F₂ plants and from F₂-plant progeny rows. If the two parental lines were allelic with regard to their genotype, then one out of four F₁ plants would be lethal yellow in crosses of known heterozygous parents. In the F₂ generation, nonsegregating families and families segregating 3 green : 1 lethal-yellow plant would be observed in a 1:2 ratio. If different genes were controlling the phenotype in T218 and T225, no lethal-yellow plants would be observed in the F₁ generation. Moreover, the F₂ generation would include nonsegregating families, families segregating 3 green : 1 lethal-yellow plant, and families segregating 9 green : 7 lethal-yellow plants in a 7:4:4 ratio. Inheritance and allelism of T362H were described previously (Palmer et al. 2000).

Molecular mapping study

'Minsoy' (*Y18Y18*) (female parent) was crossed with T225H (*Y18y18*) plants using standard soybean crossing techniques at the Bruner Farm, near Ames, Iowa, in the sum-

Fig. 1. Linkage map of the USDA – Iowa State University molecular linkage groups B2 and D2. (A) A linkage map of molecular linkage group B2 constructed from the *G. max* × *G. soja* population (Cregan et al. 1999). (B) Map position of the *Y18_1* locus, represented in this study from the cross ‘Minsoy’ (*Y18_1 Y18_1 Y18_2 Y18_2*) × T225H (*Y18_1 y18_1 y18_2 y18_2*), on molecular linkage group B2. *Satt474 and Satt560 have not been mapped in the *G. max* × *G. soja* population, but in the ‘Minsoy’ × *G. max* ‘Noir 1’ population (Cregan et al. 1999). (C) A linkage map of molecular linkage group D2 constructed from the *G. max* × *G. soja* population (Cregan et al. 1999). (D) Map position of the *Y18_2* locus, represented in this study from the cross ‘Minsoy’ (*Y18_1 Y18_1 Y18_2 Y18_2*) × T225H (*Y18_1 y18_1 y18_2 y18_2*), on molecular linkage group D2.



mer of 2000. F_1 seeds were advanced to F_2 seeds at the University of Puerto Rico – Iowa State University Soybean Nursery near Isabela, Puerto Rico. All F_1 plants exhibited normal green foliage. The F_2 seeds were planted at the Bruner Farm in May 2001. For the mapping study, 77 green F_2 plants were selected randomly from one F_2 population showing a 15 green : 1 lethal yellow segregation. The green F_2 plants were harvested individually. These F_3 seeds were planted in a sandbench in the USDA glasshouse, between September and December 2001. Segregation of green and (or) lethal-yellow plants in each F_3 line was recorded at two or three weeks after planting to identify the F_2 genotype.

SSR analysis

Soybean DNA was isolated from freeze-dried leaf tissue of parents and 77 F_2 plants from the cross between ‘Minsoy’ and *Y18y18* according to Keim et al. (1988). Simple sequence repeat (SSR) markers (Akkaya et al. 1992) were evaluated. The PCR mixture contained 50 ng of soybean genomic DNA, 1.75 mM $MgCl_2^{2+}$, 0.15 mM of sense and antisense primers, 150 μ M of each nucleotide, 1× PCR buffer, and 0.5 U *Taq* DNA polymerase (Promega, Madison, Wis.) in a total volume of 30 μ L. Cycling consisted of 45 s at 94° C, 45 s at 47° C, and 45 s at 68° C for 32 cycles on a

PTC-100™ programmable thermal controller (MJ Research, Waltham, Mass.). PCR products were run on a 2.0% w/v Agarose 3:1™ E776 (AMRESCO, Solon, Ohio) gel in TBE (0.089 M Tris–borate, 0.089 M boric acid, 0.002 M EDTA) buffer with ethidium bromide incorporated in the gel. Alternatively, PCR products were run on a sequencing gel; 8% w/v acrylamide : bis-acrylamide (29:1), 5.6 M urea, and a 0.30 volume fraction of formamide in TAE (0.04 M Tris, 0.01 M acetate, 0.001 M EDTA) buffer.

Linkage analysis

The Mapmaker 3.0 program (Lander et al. 1987) was used to construct a linkage map. In the genotype raw data of Mapmaker procedure, (i) F_2 family showing all green plants in F_3 progeny test was “–”, meaning missing data, (ii) F_2 family segregating as 3:1 ratio in F_3 progeny test was “C” meaning “not AA or BB”, and (iii) F_2 family segregating as 15:1 ratio in F_3 progeny test was “H”, including “Aa and Bb”, respectively. A LOD score of 3 was used as the lower limit for accepting linkage between two markers. Recombination frequencies were converted to map distances in centimorgans (cM) by the Kosambi function (Kosambi 1944). On the basis of two-point analysis, Mapmaker generated log-likelihood values for the most probable order.

Table 1. Segregation of SSR markers on soybean molecular linkage groups B2 and D2 in an F₂ population of a cross between 'Minsoy' (*Y18_1 Y18_1 Y18_2 Y18_2*) and T225H (*Y18_1 y18_1 y18_2 y18_2*).

MLG*	Marker	Distance [†] (cM)	Observed number				$\chi^2_{(4:8:3)}$	P
			AA [‡]	AB [‡]	BB [‡]	Total [§]		
B2	Satt577	76.3	19	40	17	76	0.30	0.86
	Satt126	60.0	22	38	17	77	0.50	0.78
	Satt304	12.8	21	47	8	76	4.47	0.11
	Satt474	4.4	21	49	7	77	6.13	0.05
	Satt534	13.4	19	43	15	77	0.22	0.90
	Satt560	30.2	24	36	17	77	1.38	0.05
D2	Satt397	21.7	26	33	18	77	3.48	0.18
	Satt389	6.9	23	30	24	77	8.08	0.02
	Satt574	2.9	26	28	23	77	9.36	0.01
	Satt543	2.2	26	29	22	77	7.83	0.02
	Sat 001	4.4	27	25	25	77	14.31	0.001
	Satt301	7.7	23	30	24	77	8.08	0.02
	Satt310	24.1	18	35	24	77	6.01	0.05
	Satt386	40.9	15	42	20	77	2.89	0.24

*Molecular linkage group.

[†]Distance (cM) from the duplicated chlorophyll-deficient loci, *y18_1 y18_1* and *y18_2 y18_2*, on MLG-B2 and D2, respectively.[‡]Genotypes: AA, 'Minsoy'; AB, heterozygote; BB, *y18_1 y18_1* or *y18_2 y18_2*.[§]Total number of 76 means that one F₂ genotype at each SSR marker locus was not identified.

Comparative mapping

Based on the soybean composite map (http://grain.jouy.inra.fr/cgi-bin/webace?seme=1&db=soybase&class=Map_Collection&object=%21Composite_Genetic_Map&display=text) and orthologous RFLP marker loci (Shoemaker et al. 1996), a comparative map between the genomic regions including the duplicated chlorophyll-deficient loci was demonstrated.

Results and discussion

Genetic study

Derivation and inheritance of T218H

'Illini' (*Y18 Y18*; female parent) was crossed to T218M (*Y18-m_*), using flowers from mostly yellow branches, in the glasshouse. All nine F₁ plants were green. In the F₂, two families were all green plants, and the other seven families segregated 2658 green : 833 lethal-yellow plants ($\chi^2_{3:1} = 2.41$, $P = 0.12$). No variegated (chimeric) plants were observed in the F₁ or F₂ generations. Twenty F₂ plants within each of the seven segregating families were single-plant threshed and evaluated as F₂-plant progeny rows. The F₂ family segregation was 95 segregating : 45 nonsegregating ($\chi^2_{2:1} = 0.09$, $P = 0.77$). The number of F₃ plants in segregating families was 4750 green : 1541 lethal-yellow plants ($\chi^2_{3:1} = 0.85$, $P = 0.36$). The F₃ data confirmed the single recessive gene inheritance for lethal-yellow plants in T218H, its original genetic background (Palmer 1987).

The failure of two F₁ plants to segregate in the F₂ for green and lethal-yellow plants was not unexpected. In general, segregation patterns of chimeric plants manifest a relationship between sector phenotype and gamete genotype. When chimeric plants are used in cross-pollinations and the lethal-yellow trait is a single recessive gene, flowers on yellow branches may produce all green F₂ progeny, green and

chimeric F₂ progeny, or about 3 green : 1 lethal-yellow F₂ progeny.

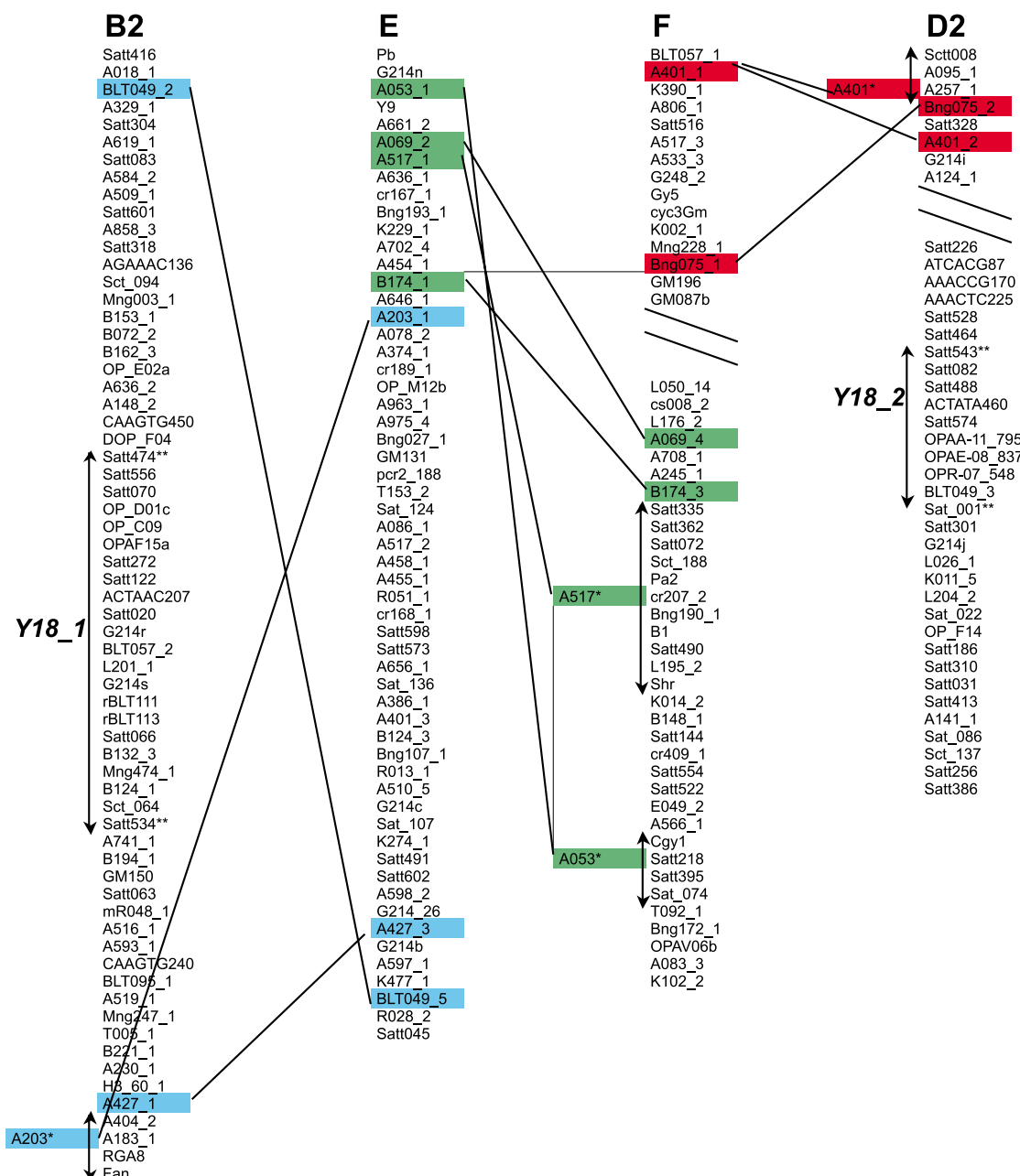
The F₂ segregation, in segregating families, for the reciprocal crosses of T218H (*Y18 y18*) by PI 437477B (*Y18 Y18*) were 904 green : 56 lethal-yellow plants ($\chi^2_{15:1} = 0.28$, $P = 0.59$, which was a good fit to a 15:1 ratio). The overall F₂-family segregation was 48 nonsegregating : 26 segregating (3:1) : 26 segregating (15:1) ($\chi^2_{7:4:4} = 0.50$, $P = 0.78$). The lethal-yellow trait from T218H (*Y18 y18*) was inherited as a duplicate factor recessive in the PI 437477B cross.

Derivation and inheritance of T225H

'Lincoln' (*Y18 Y18*) (female parent) was crossed by T225 (*y18 y18*) plants in the growth chamber. The F₁ plant was green and the F₂ segregated 312 green : 88 lethal-yellow plants ($\chi^2_{3:1} = 1.92$, $P = 0.17$). No variegated (chimeric) plants were observed in the F₁ or F₂ generations. In its original background of 'Lincoln', *y18* from T225 was inherited as a single recessive gene. One hundred twenty green F₂ plants were single-plant threshed and evaluated as F₂ progeny rows. The F₂ family segregation was 83 segregating : 37 nonsegregating ($\chi^2_{2:1} = 0.34$, $P = 0.56$). The number of F₃ plants in segregating families was 1782 green : 608 lethal-yellow ($\chi^2_{3:1} = 0.25$, $P = 0.62$). The F₃ data confirmed the single recessive gene for lethal-yellow plants in T225, its original genetic background (Sheridan and Palmer 1975).

The F₂ segregation, in segregating families, for the reciprocal crosses of 'Minsoy' with T225H was 425 green : 27 lethal-yellow plants ($\chi^2_{15:1} = 0.56$, $P = 0.81$), and for the reciprocal crosses of T225H with PI 437477B was 452 green : 28 lethal-yellow plants ($\chi^2_{15:1} = 0.14$, $P = 0.74$). The F₂-family segregation for the PI 437477B cross (*Y18 Y18*) was 34 nonsegregating : 17 segregating (3:1) : 19 segregating (15:1) ($\chi^2_{7:4:4} = 0.55$, $P = 0.76$). The lethal-yellow trait from T225H (*Y18 y18*) was inherited as a duplicate factor recessive both in the 'Minsoy' (*Y18 Y18*) cross and the PI 437477B (*Y18 Y18*) cross.

Fig. 2. Comparative map among soybean molecular linkage groups B2, E, F, and D2. Marker order was based on the composite map (http://grain.jouy.inra.fr/cgi-bin/webace?seme=1&db=soybase&class=Map_Collection&object=%21Composite_Genetic_Map&display=text). Homoeologous RFLP markers were combined by the dotted lines (Shoemaker et al. 1996). Asterisk indicates map positions of A203 on molecular linkage group B2, A517, and A053 on molecular linkage group F, and A401 on molecular linkage group D2 were based on USDA – Iowa State University map (Cregan et al. 1999.). Double asterisk indicates *Y18_1* located between SSR markers Satt474 and Satt534, and *Y18_2* was located between SSR markers Satt543 and Sat_001, respectively.

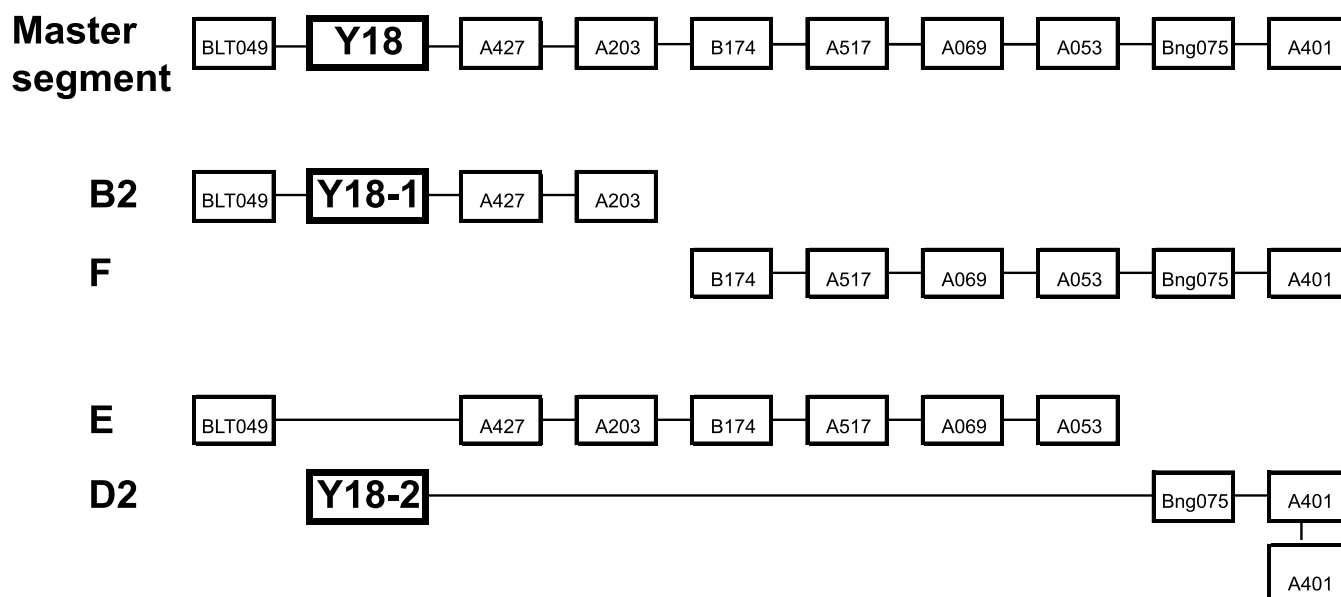


Allelism tests of T218H and T225H

Reciprocal cross-pollinations were made by using green plants of T218H and T225H (Hatfield and Palmer 1986). The homogeneity χ^2 test indicated that data were homogeneous and pooled. Individual plant identity of the parents was maintained and self-pollinated seed was planted to determine the genotype of the parents, i.e., AA or Aa. The F_1 plant color segregation from crossing two heterozygous genotypes was 33 green : 10 lethal yellow ($\chi^2_{3:1} = 0.07$, $P =$

0.79). Thirty F_1 green plants were single-plant threshed and evaluated for F_2 plant color segregation. The family segregation was 22 segregating : 8 nonsegregating ($\chi^2_{2:1} = 0.60$, $P = 0.44$). The number of F_2 plants in segregating families from the reciprocal crosses was 5242 green : 1693 lethal yellow ($\chi^2_{3:1} = 1.28$, $P = 0.26$). Five green F_2 plants within each of the segregating families were single-plant threshed and a total of 110 plants were evaluated as F_2 progeny rows. The F_2 family segregation was 68 segregating : 42 nonsegregating

Fig. 3. Graphical representation of gene content of each chromosomal segment on soybean molecular linkage groups B2, F, E, and D2. Homoeologous RFLP clones (Shoemaker et al. 1996) and duplicated chlorophyll-deficient mutant loci, *Y18_1* and *Y18_2*, are presented.



($\chi^2_{2:1} = 1.16$, $P = 0.28$). The number of F_3 plants in segregating families from the reciprocal crosses was 2682 green : 854 lethal yellow ($\chi^2_{3:1} = 1.36$, $P = 0.24$).

If T218H and T225H are nonallelic, all F_1 plants from T218H \times T225H were expected to be green. If they are allelic, crosses between heterozygous genotypes are expected to segregate 3 green : 1 lethal yellow. Our data indicated that T218H and T225H are allelic. Both F_2 and F_3 family segregation were in agreement with the expected 2 segregating : 1 nonsegregating ratio. Furthermore, in segregating F_2 and F_3 families, segregation ratios of green : lethal-yellow plants were in agreement with the expected 3:1 ratio. A ratio of 9 green : 7 lethal-yellow plants, which would indicate two nonallelic loci, was not observed. Collectively, these data confirm the allelism of these two mutants. The event(s) that produced the unstable (mutable) allele in T218M ('Illini') and T225M ('Lincoln') occurred in different cultivars, probably during different years and at two locations (1952 at Urbana, Ill., for 'Illini' and before 1955 at Ames, Iowa, for 'Lincoln'). The conclusion is that they represent two independent events.

Genetic Type Collection numbers and gene symbols are T218M (*Y18-m_y18_2y18_2* (Urbana)), T218H (*(Y18_1 y18_1 y18_2 y18_2)* (Urbana)), T225M (*Y18-m_y18_2 y18_2*) and T225H (*Y18_1 y18_1 y18_2 y18_2* (Ames 1)). The dash (–) in T218M and Y225M signifies *Y18-m*, *Y18*, or *y18*.

Molecular mapping study

The segregation of normal vs. lethal-yellow plants in the F_2 population in the 'Minsoy' \times T225H (*Y18 y18*) cross was 123:9 and followed a 15:1 ratio ($\chi^2 = 0.07$; $P = 0.79$). A total of 77 green plants were randomly selected from among the 123 green plants in the F_2 generation to determine the genotype. The F_2 family segregation was 37 nonsegregating :

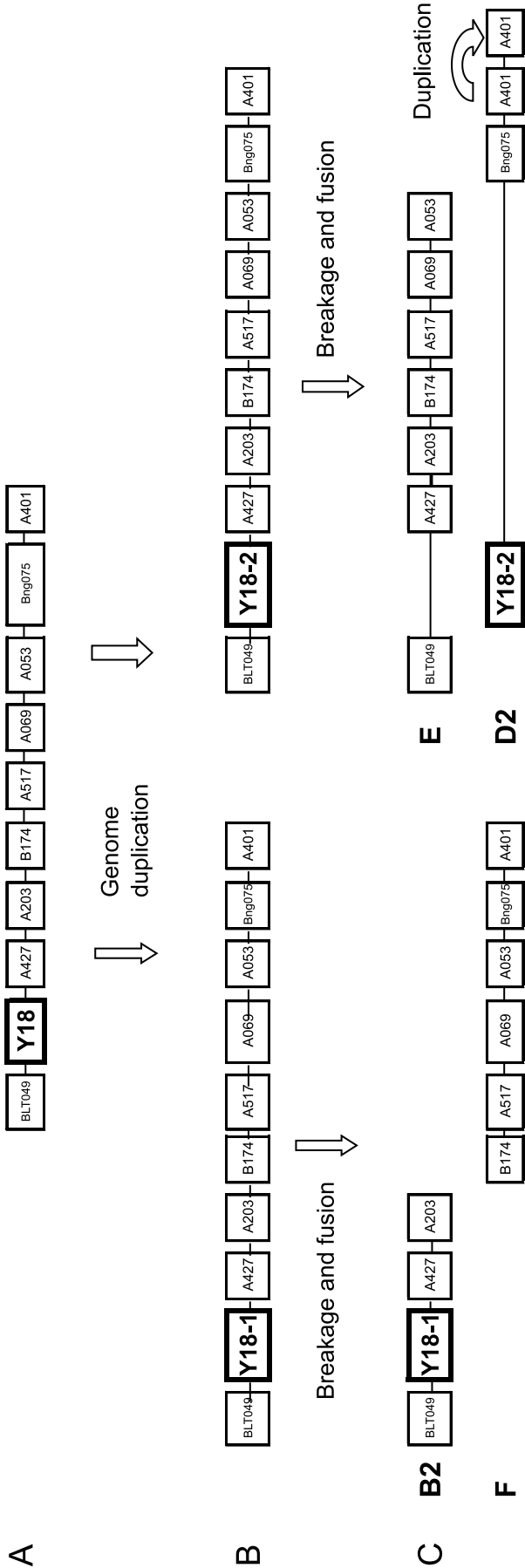
17 segregating (3:1) : 23 segregating (15:1), and followed a 7:4:4 ratio ($\chi^2 = 1.21$, $P = 0.54$).

Initial screening of the F_2 population was conducted by selecting several SSR markers from each linkage group (Cregan et al. 1999). The markers were chosen to divide each linkage group into segments of less than 30 cM. Two hundred six SSR markers were tested to detect polymorphism between parents. Among them, 86 (41.7%) showed polymorphism between the parents. SSR markers Satt304 on molecular linkage group B2 and Satt389 on molecular linkage group D2 were identified as linked to the two loci (LOD = 3.90 and LOD = 3.60, respectively).

From molecular linkage group B2, 12 additional markers, Satt577, Satt126, Satt467, Satt168, Satt416, Satt083, Satt601, Satt318, Satt534, Sct_034, Satt474, and Satt560 (Cregan et al. 1999) were screened between the parental lines. We detected polymorphisms using the following SSR markers: Satt577, Satt126, Satt534, Satt474, and Satt560. On the basis of LOD scores generated from the Mapmaker program, the *y18* locus was linked to SSR marker Satt474 with LOD score of 4.83. The most likely order of markers is shown in Fig. 1. The *y18* locus, designated *y18_1*, was located between Satt474 and Satt534, and linked to each by 4.4 and 13.4 cM, respectively. Both Satt577 and Satt126 segregated independently of *y18_1*. No polymorphisms were observed using SSR markers: Satt467, Satt168, Satt416, Satt083, Satt601, Satt318, and Sct_034.

From molecular linkage group D2, 21 additional markers, Satt135, Satt458, Satt014, Satt498, Satt486, Satt372, Satt002, Satt154, Satt582, Satt443, Satt397, Satt208, Satt543, Satt574, Sat_001, Satt301, Sat_022, Satt310, Satt386, Satt328, and Sat_092 (Cregan et al. 1999), were screened between parental lines. We detected polymorphisms using the following SSR markers: Satt397, Satt543, Satt574, Sat_001, Satt301, Satt310, and Satt386. On the basis of LOD scores generated from Mapmaker pro-

Fig. 4. A possible genomic evolution model of four current chromosomal segments on soybean molecular linkage groups B2, D2, E, and F from the proposed ancestral chromosome segment. Single genome duplication and a series of rearrangements are included in this model. (A) A master segment includes a single copy of all 10 homologous gene and (or) RFLP clones: Y18, BLT049, A427, A203, B174, A517, A069, A053, Bng075, and A401. (B) Genome duplication maintains a gene content in master segment. (C) Chromosome breakage and fusion with other chromosomal segments as observed in current molecular linkage groups B2, E, F, and D2, respectively. It is likely that segmental inversion on four segments and duplication of A401 on molecular linkage group D2 occurred after duplication of a master segment.



gram, the other locus was linked to SSR markers Satt574, Satt543, and Sat_001 with LOD scores of 4.27, 5.07, and 3.02, respectively. The most likely order of markers is shown (Fig. 1). The other *y18* locus, designated *y18_2*, was located between Satt543 and Sat_001, and linked to each by 2.2 cM and 4.4 cM, respectively. No polymorphisms were observed using the following SSR markers: Satt135, Satt458, Satt014, Satt498, Satt486, Satt372, Satt002, Satt154, Satt582, Satt443, Satt208, Sat_022, Satt328, or Sat_092.

Segregation ratios of most tested SSR markers on molecular linkage group B2 and D2 provided good fits to a 4 : 8 : 3 ratio (Table 1), supporting the data that these SSR markers were linked to each duplicated chlorophyll-deficient locus.

Comparative mapping of duplicated chlorophyll-deficient *y18* loci

Multiple homoeologous regions have been identified throughout the soybean genome (Shoemaker et al. 1996). The most-parsimonious explanation for the presence of the duplicate *y18_1* and *y18_2* loci is that they are a result of the previously postulated whole-genome polyploidization event (Shoemaker et al. 1996). Shoemaker et al. (1996) demonstrated that (i) molecular linkage groups B2 and E carry the homoeologous genomic sequences hybridizing with three common RFLP clones: A203, A427, and BLT049; (ii) molecular linkage groups E and F carry the homoeologous genomic sequences hybridizing with four common RFLP clones: A053, A069, A517, and B174; (iii) molecular linkage groups F and D2 carry the homoeologous genomic sequences hybridizing with two common RFLP clones: A401 and Bng075, respectively. Combined with the soybean composite map (http://grain.jouy.inra.fr/cgi-bin/webace?seme=1&db=soybase&class=Map_Collection&object=%21Composite_Genetic_Map&display=text), we constructed the comparative map among molecular linkage groups B2, E, F, and D2, including the map position of the duplicate loci (Fig. 2). The comparative map demonstrated that the chromosomal segments including the duplicated *y18_1* and *y18_2* loci were not directly homoeologous genomic regions.

Evolutionary history of four segments on molecular linkage groups B2, E, F, and D2

Genomic contents of the functional *y18_1* and *y18_2* loci and hybridization with nine RFLP clones, BLT049, A427, A203, B174, A517, A069, A053, Bng075, and A401, on molecular linkage group B2, E, F, and D2 are illustrated in Fig. 3. These genomic contents suggested a possible evolutionary relationship between the two pairs of homoeologous genomic regions. The first pair includes the two segments from molecular linkage groups B2, which carries the genomic region hybridizing with BLT049, A427, A203, and the *y18_1* locus, and F, which carries the genomic region hybridizing with B174 A517 A069, A053, Bng075, and A401. The second pair includes the remaining two segments from molecular linkage group E, which carries the genomic region hybridizing with BLT049, A427, A203, B174, A517, A069, and A053; D2, which carries the *y18_2* locus; and the genomic region hybridizing with Bng045 and A401.

An explanation can be proposed for the evolution of these genomic segments. The following is the most-parsimonious explanation for the evolutionary history of these four genomic

segments based on the possible ancestral chromosomal segment (master segment) including all genomic segments hybridizing with nine common RFLP clones and the two loci (Fig. 4): (i) a master segment was duplicated; (ii) each master segment was divided into two segments to carry the different combinations of genomic content; (iii) the four divided genomic segments were fused with other genomic segments from independent chromosomes, respectively. Single genic or small regions, including the genomic content hybridizing with A401, were then duplicated on the current molecular linkage group D2 after master segment duplication.

In the future, we need to study duplicated genes and (or) segments using a molecular genetic approach. If homoeologous genomic DNA sequences of the two loci themselves that hybridize with each RFLP clone are compared, the time of duplication event of these genes could be estimated via molecular time. The proposed duplication of this master segment on the recent polyploidization event in soybean could also be clarified.

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